Kidney International, Vol. 53 (1998), pp. 154–158

Functional quantitative analysis of the genome in cultured human mesangial cells

Technical Note

Yoshinari Yasuda, Toshio Miyata, Masaomi Nangaku, Yoshiyasu Iida, Kenji Maeda, Kiyoshi Kurokawa, and Kousaku Okubo

Institute of Medical Science and Department of Medicine, Tokai University School of Medicine, Isehara; Department of Internal Medicine, Nagoya University School of Medicine, Nagoya; and Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan

Functional quantitative analysis of the genome in cultured human mesangial cells. For normal physiological function, each cell tightly regulates gene expression in a specific fashion so that critical proteins are synthesized in a well-coordinated manner. Therefore, it is very important to uncover which genes are expressed in specific cells. Recent technological advances combined with rapid large-scale DNA sequencing and computerized data processing have allowed us to investigate the expression levels of a variety of transcripts in the mesangial cells, a target of injury in many forms of glomerulonephritis. Utilizing a large scale sequencing of a 3′-directed cDNA library, which allows us to avoid variable cloning efficiencies reflecting the size of cDNA, we investigated expression profiles of various molecules in cultured human mesangial cells. Among the 1,193 sequenced clones, 688 (57.7%) appeared more than once (redundant sequence group), representing 203 different species. Thirty-nine of these appeared more than three times. The most abundant mRNA was that of fibronectin, which consisted of 3.9% of the total mRNA population. Except for mitochondrial or ribosomal genes, calcyclin came next (2.5%), followed by two cytoskeletal genes, γ-actin gene and calpactin 1 light chain gene, in addition to an amyloid precursor protein homolog (0.7%). In conclusion, we performed a molecular biological quantification of transcripts in mesangial cells. Fibronectin was the most abundantly expressed, followed by calcyclin, γ-actin, calpactin 1 light chain, and an amyloid precursor protein homolog. We also discovered some candidate genes specific for human mesangial cells. The expression profile of the transcripts serves as an important tool in understanding the biological properties of mesangial cells.

There are about 60 trillion (6×10^{13}) cells in the human body. Despite the wide variety in cell phenotypes, all the cells share essentially identical genomic DNA, being the faithful copy of that originally carried in the fertilized egg [1]. Expression of these genes is tightly regulated by the cell lineage and the signals that the cell receives in order for normal physiological function. Therefore, it is very important to uncover which genes are expressed in specific cells.

Key words: expression profile, 3′-directed cDNA library, DNA sequencing, genome analysis.

Received for publication July 2, 1997
and in revised form August 28, 1997
Accepted for publication August 28, 1997

Conventional biochemical and molecular biological approaches such as metabolic labeling, Western blot and Northern blot analyses have limitations in comparing expression levels of multiple molecules, although they are useful in investigating temporal profiles of expression levels of the same protein or gene, because each molecule requires a unique antibody or probe. One must use as many different antibodies or probes as the number of molecules chosen to investigate. Furthermore, the affinities of these antibodies or probes are different from each other, which hampers comparison between various molecules. Some molecular biological approaches such as subtraction cloning and differential hybridization allowed us to compare libraries from two different sources and detect cell- or organ-specific genes. However, no quantitative information about expression levels of specific and non-specific genes can be obtained. No more than two libraries can be compared at one time using these methods, either.

We utilized recent technological advances combined with rapid large-scale DNA sequencing and computerized data processing in order to compare the expression levels of a variety of transcripts in specific cells at the same time. Utilizing a large scale sequencing of a 3′-directed cDNA library, we investigated expression profile of various genes of mesangial cells. This is the first report of functional quantitative analysis of the genome in glomerular cells.

METHODS

Primary culture of human mesangial cells

Human glomerular mesangial cells were isolated from a normal human kidney obtained from surgical specimens obtained during nephrectomy in a 58-year-old male with kidney tumor. The renal cortex was separated, minced, and passed through a series of progressively smaller pore sized seives under sterile conditions. Glomeruli, trapped by sieves with a pore size of 75 to 200 μm, were washed and incubated with 100 μg/ml collagenase (Washington Biochemical) at 37°C for 20 minutes. After washing, glomeruli were resuspended in medium 199 (Gibco BRL, Gaithersburg, MD, USA) containing 25 mM Hepes, 10% Nu-serum (Collaborative Biomedical Products, Bedford, MA, USA) and antibiotics (10 mg/ml of penicillin, streptomycin, and fungizone), and were incubated in a 5% CO_2 incubator. Mesangial cells at

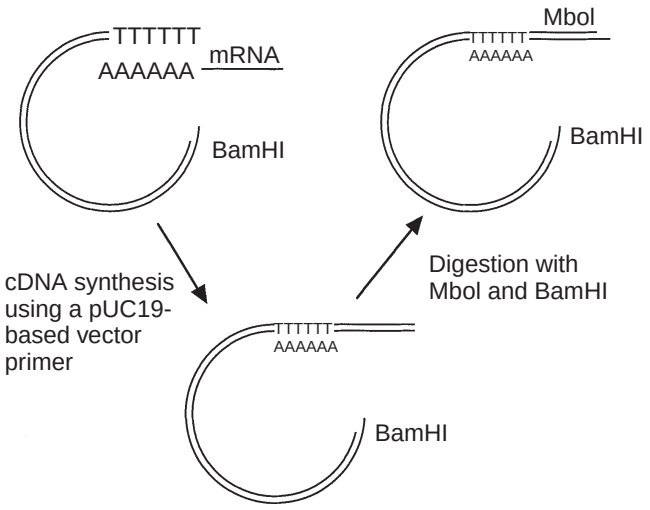

Fig. 1. Schematic representation of construction of the 3′-directed cDNA library. Cytoplasmic polyA$^+$ DNA was used as a template for the cDNA synthesis utilizing a pUC19-based vector-primer DNA. After second strand synthesis, the cDNA moiety and the vector were cleaved by MboI and BamHI, followed by circularization and ligation. Cohesive end of BamHI digestion can adhere to MboI end.

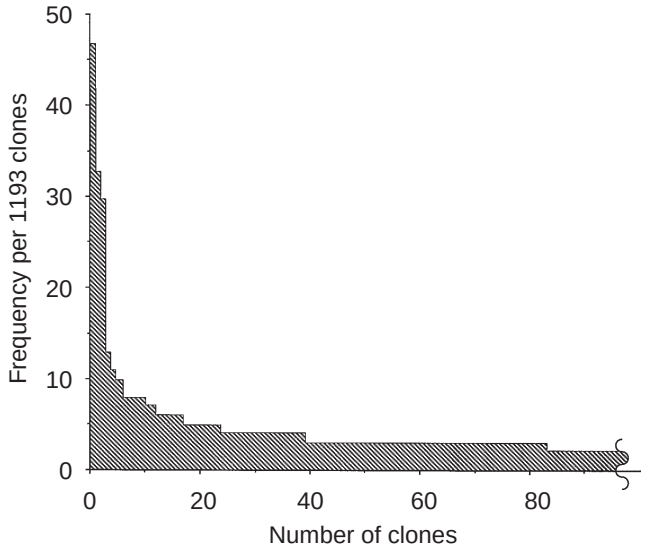

Fig. 2. Expression profile of genes in cultured human mesangial cells. The frequencies of the appearance of each cDNA species in the randomly sequenced 1,193 clones are shown. Species are arrayed in the order of their frequencies of appearance.

passage 3 were characterized by a series of criteria [2], including typical morphologic features, tolerance to trypsin, puromycin, and D-valine, positive immunostaining for actin (Zymed Laboratories, San Francisco, CA, USA), anti-very late antigen (VLA)-1,3,5 (Immunotech), and negative immunostaining for Factor VIII (Dako, CA, USA).

Isolation of mRNA from human cultured mesangial cells

Total RNA was isolated from human mesangial cells at passage 6 using the guanidine isothiocyanate (GTC) method. In brief, confluent culture of mesangial cells in the culture medium containing serum described above were washed with phosphate-buffered saline (PBS), lysed in 5.5 mM GTC solution. The DNA was sheared by passing through a 18 gauge needle. Nuclei and other cell debris were precipitated by centrifuge at 5,000 × g for 90 seconds. The supernatant was gently overlayed onto the cushion of cesium trifluoroacetate (CsTFA) solution and centrifuged at 125,000 × g for 24 hours at 15°C. The RNA pellet was dissolved in TE buffer. PolyA$^+$ RNA was isolated by oligo dT-cellulose column (Pharmacia, Tokyo, Japan) according to the manufacturer's protocol.

Construction of the 3′-directed cDNA library

Poly A$^+$ RNA was used as a template for the cDNA synthesis using a pUC19-based vector primer [3]. This vector-primer DNA has HincII and T-tailed PstI ends, and was dam-methylated at the MboI sites (GATC). After the second strand synthesis, the cDNA moiety and the single BamHI site in the lacZ gene of the vector were cleaved by MboI and BamHI, respectively, followed by circularization and ligation at low DNA concentration. A small portion of the ligation mixture was transformed into *E. coli*. The resulting transformants were selected randomly and were lysed individually by brief boiling. The cDNA insert moiety was amplified by paired PCR with primers (5′-TGTAAAACGACGGCCAGT-3′ and 5′-ACCATGATTACGCCAAGCTTG-3′) flanking the pUC19 cloning site. The resulting short double-stranded DNA was utilized for the cycle sequencing reaction and was analyzed using an autosequencer.

RESULTS

cDNA library construction and sequencing

Cultured human mesangial cell mRNA was prepared and used as a template for cDNA synthesis. The cDNA moiety was selectively cleaved by MboI, and the 3′-directed regional cDNA library was constructed by religation (Fig. 1) and transformation into *E. coli*. The library was not amplified in order to make them faithfully represent the original mRNA populations.

We randomly chose 1,836 transformant colonies, the cDNA moieties of which were amplified by PCR and subjected to automated cDNA sequencing. Since the sequences at the 3′-region are unique, sequencing data from about 150 to 300 nucleotides, which are called the "gene signature," were sufficient to characterize the gene.

Among the 1,836 clones analyzed, those that had inserts shorter than 20 bp were excluded from further analysis. The sequence similarities of the remaining 1,193 clones were compared with each other and with the DNA databank GenBank utilizing the FASTA program [4]. We regarded two sequences as identical when the overlapping region that had more than 90% homology started at the first base in both and exceeded 90% of the length of the shorter one of the pair.

Quantitative analysis of expressed genes

The frequency distribution of cDNA species is demonstrated in Figure 2. Among the 1,193 sequenced clones, 688 (57.7%) appeared more than once (redundant sequence group), representing 203 different species. Thirty-nine of these appeared more than three times.

By re-association kinetics mRNA in mammalian cells is categorized into high, middle, and low abundance classes [5]. The

Table 1. Abundant clones in cultured mesangial cells

Frequency/ 1193 clones	Gene signature	Accession number	Name of the gene
47	2105	X02761	fibronectin
33	744	V00710	mit. genes for several tRNAs
30	1919	J02763	calcyclin
13	4444	M81757	S19 rib. protein
11	688	L00016	mit. trnas and partial proteins
10	500	D14530	mRNA for rib. protein
8	114	X04098	cytoskeletal gamma actin
8	335	M36072	rib. protein L7a (surf 3) large
8	418	D14531	human homologue of rat rib. protein L9
8	1608	S60099	amyloid precursor protein homolog
7	521		Unknown
7	3867	M81457	calpactin 1 light chain
6	96		Unknown
6	155	M11948	promyelocytic leukemic cell mRNA, clones of pHH58 and pHH81
6	285		Unknown
6	363	D14696	identical sequence to HepG2 and mRNA for ORF
6	512	M22146	scar protein
5	211	X67247	rib. protein S8
5	356	X53777	L23 mRNA for putative rib. protein
5	385		Unknown
5	504	M14328	alpha enolase
5	882		Unknown
5	2123		Unknown
5	9344		Unknown

Some properties of the most abundant 24 clones are shown. Abbreviations are: rib, ribosomal; mit, mitochondrial.

highly abundant mRNA is proposed to consist of a few species and to cover 0 to 20% of the total mRNA. The middle abundant class covers some 30 to 40% of the total mRNA, consisting of a few hundred species, and the low abundant class covers about 50% of the total mRNA consisting of about 10,000 to 30,000 species. We observed four genes of high abundance, each of which exceeded 1%, 80 genes in the middle abundance class each of which exceeded 0.2%, and about 620 genes in the low abundance class, at ratios of 10, 30 and 60%, respectively.

Some properties of the most abundant 24 clones in mesangial cells are shown in Table 1. The most abundant mRNA was that of fibronectin (FN), which consisted of 3.9% of all the mRNA population. Except for mitochondrial or ribosomal genes, calcyclin came next (2.5%), followed by two cytoskeletal genes, γ-actin gene and calpactin 1 light chain gene, in addition to amyloid precursor protein homolog (0.7%). The "expression profiles" in mesangial cells were apparently different from those obtained from other libraries (Table 2), which confirmed specificity of "expression profiles" in different cells and organs.

DISCUSSION

We measured "expression profiles" of genes in mesangial cells, which allowed us to perform functional analyses of the genome in mesangial cells. This was achieved through sequencing randomly selected clones from a non-biased cDNA library.

This library must faithfully represent the abundance of gene transcripts in the original mRNA population, which gives us quantitative information of expressed genes. In a full-length

Table 2. Frequencies of 10 most abundant clones in other cDNA libraries

Lane	1	2	3	4	5	6	7	8	9	10
Mesangium	47	33	30	13	11	10	8	8	8	8
Lung	10	6	3	1	16	2	3	3	0	0
HepG2	0	0	0	6	0	6	1	1	5	0

Lane 1, FN; lane 2, mitochondrial genes for several tRNA; lane 3, calcyclin; lane 4, S19 ribosomal protein; lane 5, mitochondrial trnas and partial proteins; lane 6, ribosomal protein; lane 7, gamma actin; lane 8, ribosomal protein L7a (surf 3) large; lane 9, human homolog for rat ribosomal protein L9; lane 10, amyloid precursor protein homolog. For more details of each molecule, see Table 1.

cDNA library the 5′-termini of the cDNAs for the same transcripts often have different sequences due to the partial degradation of mRNA or incomplete first strand synthesis. Furthermore, the nucleotide sequences of their 3′-termini can not be determined easily by chain termination method with universal primer because of the slippage in the primer extension at polyA$^+$. A random-primed cDNA library, which is utilized to construct expressed sequence tag (EST) database, is useful to discover new genes [6–9]. However, it cannot be used to obtain gene signatures, since it is not possible to tell whether two partial sequences are from different parts of a single gene or from different transcripts. Therefore, we employed a 3′-directed regional cDNA library [10, 11]. cDNA cleaved by MboI, which cuts on average a few hundred bases from the polyA tail of each cDNA, were used. We can avoid variable cloning efficiencies reflecting the size of cDNA with this approach. The sequences at the 3′-region are unique, and sequencing data from about 200 to 300 bp are sufficient to characterize the gene. Redundancy of the same sequence represents the abundance of corresponding transcript in cells. The list describing the gene expressed in a given cell along with their relative activities of transcription, termed an "expression profile," is biologically significant, because quantitative analysis of the results gives us the pattern of gene expression in a cell in addition to sequence data. "Expression profiles" of genes have been published in liver cells, lung cells, and a cell line derived from hepatocellular carcinoma (HepG2) [10, 11], and this is the first report of "expression profiles" of kidney cells.

To characterize the expression profiles of genes in a given cell type, random sequencing about 1,000 clones is sufficient, because a significant fraction of middle and highly abundant cell-specific genes can be identified [12]. We sequenced 1,193 clones, which we judged to be enough.

Our quantitative analysis of expressed cDNA species showed that mRNA of FN was the most abundant in mesangial cells. Fibronectin is a high molecular weight glycoprotein that is present in two different forms. Cellular FN (cFN) is present on the surface of many cells where it plays an important role in organizing the extracellular matrix, the maintenance of cell morphology and behavior, and cell adhesion [12, 13]. Plasma FN (pFN) is abundant in body fluids and is produced by alternative splicing of precursor mRNA from the same gene. Immunohistochemical and immunofluorescence studies had shown FN predominantly in the normal mesangium [14–20], and immunoelectron microscopic studies had demonstrated the localization of FN predominantly in the mesangial matrix, especially at the interface between the

endothelial and mesangial cells [21]. Still, it had remained controversial whether FN in the mesangial area was deposited pFN or locally produced cFN. However, recently it was shown that FN observed in the mesangium was produced locally in tissues utilizing specific monoclonal antibodies [22]. Fibronectin is also reported to be increased in the mesangial area of various glomerulopathies of humans [15, 16, 18–20, 23–26]. Radiolabeled-methionine incorporation studies utilizing cultured rat mesangial cells demonstrated that FN accounted for 2.1% of total protein synthesis [27]. This value is close to our estimation of the amounts of FN mRNA. The massive amount of FN mRNA supports previous speculations that FN plays an important role in physiology of mesangial cells.

Interestingly, the second most abundant gene except for mitochondrial or ribosomal ones was calcyclin. Calcyclin, which is also called S100A6, is a member of the S100 family of Ca binding proteins and contains two "EF-hand" type domains [28, 29]. Calcyclin was originally discovered in the studies of the mechanisms by which growth factors signal quiescent cells to enter the cell cycle [30]. To date, no definite function of calcyclin has been established. Potential functions include intracellular signal transduction and regulation of cell cycle [31]. So far, there have not been any studies on roles of calcyclin in glomerular cells. Immunohistochemical studies have shown that mammalian calcyclin is mainly present in the fibroblasts and epithelial cells but not in the glomerular mesangial cells [32]. The reason for this discrepancy is unclear, but it should be noted that a potential function of calcyclin is the regulation of cell proliferation. We constructed our cDNA library utilizing rapidly growing mesangial cells cultured in the media with serum. Mesangial cells *in vivo* are known to acquire new phenotypes, which are characteristic features of cultured mesangial cells, when they are activated and proliferate [33, 34]. Calcyclin may be expressed by mesangial cells only when they are activated and can be an important regulator of mesangial cell functions. Thus, it remains to be elucidated how precisely the data of this study employing cultured mesangial cells represent the physiological properties of mesangial cells *in vivo*. We performed *in situ* hybridization studies of human kidney samples using a probe obtained from one unknown clone, which demonstrated a strong signal specifically in the mesangial area (unpublished observation). This suggests that the expression profiles of some clones obtained in this study represent those observed *in vivo*.

Besides getting "expression profiles," we obtained clones that code unknown genes, which can be mesangium-specific. In order to find cell-specific genes, several different approaches have been employed, such as subtraction cloning and differential hybridization. However, these methods allow us to compare libraries of no more than two different sources. The biggest advantage of our system is that we can discover truly specific genes by comparing "expression profiles" from multiple tissues and cells at one time.

In conclusion, we performed a molecular biological quantification of transcripts in mesangial cells. FN was the most abundantly expressed, followed by γ-actin, calpactin 1 light chain, and amyloid precursor protein homolog. We also discovered some candidate genes specific for human mesangial cells. The expression profile of the transcripts serves an important tool in understanding the biological properties of mesangial cells. We are currently cloning and investigating these mesangium-specific genes.

ACKNOWLEDGMENTS

This study was supported by a grant from Research for the Future Program of the Japan Society for the Promotion of Science (96L00303).

Reprints requests to Toshio Miyata, M.D., Ph.D., Institute of Medical Science and Department of Medicine, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-11, Japan.
E-mail: t-miyata@is.icc.u-tokai.ac.jp

APPENDIX

Abbreviations used in this paper are: CsTFA, cesium trifluoroacetate; EST, expressed sequence tag; FN, fibronectin; GTC, guanidine isothiocyanate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

REFERENCES

1. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS: Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810–813, 1997
2. Striker GE, Striker LJ: Glomerular cell culture. *Lab Invest* 53:122–132, 1985
3. Norrander J, Kampe T, Messing J: Construction of improved M13 vectors using oligo deoxynucleotide directed mutagenesis. *Gene* 26: 101–106, 1983
4. Pearson WR, Lipman DJ: Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 85:2444–2448, 1988
5. Bishop JO, Morton JG, Rosbash M, Richardson M: Three abundance classes in Hela cell messenger RNA. *Nature* 250:199–204, 1974
6. Nangaku M, Shankland SJ, Kurokawa K, Bomsztyk K, Johnson RJ, Couser WG: Cloning of a new human gene with short consensus repeats using EST database. *Immunogenetics* 46:99–103, 1997
7. Lesage F, Guillemare E, Fink M, Duprat F, Lazdunski M, Romey G, Barhanin J: TWIK-1, a ubiquitous human weakly inward rectifying K^+ channel with a novel structure. *EMBO J* 15:1004–1011, 1996
8. Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF, Kerlavage AR, McCombie WR, Venter JC: Complementary DNA sequencing: Expressed sequence tags and human genome project. *Science* 252:1651–1656, 1991
9. Adams MD, Dubnick M, Kerlavage AR, Moreno R, Kelley JM, Utterback TR, Nagle JW, Fields C, Venter JC: Sequence identification of 2375 human brain genes. *Nature* 355:632–634, 1992
10. Matsubara K, Okubo K: cDNA analyses in the human genome project. *Gene* 135:265–274, 1993
11. Okubo K, Hori N, Matoba R, Niiyama T, Fukushima A, Kojima Y, Matsubara K: Large scale cDNA sequencing for analysis of quantitative and qualitative aspects of gene expression. *Nature Gene* 2:173–179, 1992
12. Couchman JR, Beavan LA, McCarthy KJ: Glomerular matrix: Synthesis, turnover and role in mesangial expansion. *Kidney Int* 45:328–335, 1994
13. Mene P, Simonson MS, Dunn MJ: Physiology of the mesangial cell. *Physiol Rev* 69:1347–1424, 1989
14. Oberley TD, Mosher DF, Mills MD: Localization of fibronectin within the renal glomerulus and its production by cultured glomerular cells. *Am J Pathol* 96:651–662, 1979
15. Oomura A, Nakamura T, Arakawa M, Ooshima A, Isemura M: Alterations in the extracellular matrix components in human glomerular diseases. *Virchows Archiv A Pathol Anat* 415:151–159, 1989
16. Weiss MA, Ooi BS, Ooi YM, Engvall E, Ruoslahti E: Immunofluorescent localization of fibronectin in the human kidneys. *Lab Invest* 41:340–347, 1979
17. Mounier F, Foidart J-M, Gubler M-C: Distribution of extracellular matrix glycoproteins during normal development of human kidney: An immunohistochemical study. *Lab Invest* 54:394–401, 1986
18. Linder E, Miettinen A, Törnroth T: Fibronectin as a marker for the glomerular mesangium in immunohistology of kidney biopsies. *Lab Invest* 42:70–75, 1980
19. Dixon AJ, Burns J, Dunnill MS, McGee JO: Distribution of fibronectin in normal and diseased human kidneys. *J Clin Pathol* 33:1021–1028, 1980

20. Ikeya M, Nagase M, Honda N: Intraglomerular distribution of fibronectin in primary glomerular diseases. *Clin Nephrol* 24:53–59, 1985
21. Courtoy PJ, Kanwar YS, Hynes RO, Farquhar MG: Fibronectin localization in the rat glomerulus. *J Cell Biol* 87:691–696, 1980
22. Laitinen L, Vartio T, Virtanen I: Cellular fibronectins are differentially expressed in human fetal and adult kidney. *Lab Invest* 64:492–498, 1991
23. Altunkova I, Minkova V, Belovezhdov N: Role of fibronectin in immune glomerulonephritis. *Nephron* 63:438–444, 1993
24. Pettersson EE, Colvin RB: Cold-insoluble globulin (fibronectin, LETS protein) in normal and diseased human glomeruli. Papain-sensitive attachment to normal glomeruli and deposition in crescents. *Clin Immunol Immunopathol* 11:425–436, 1978
25. Büyükbabani N, Droz D: Distribution of the extracellular matrix components in human glomerular lesions. *J Pathol* 172:199–207, 1994
26. Scheinman JI, Fish AJ, Matas AJ, Michael AF: The immunohistopathology of glomerular antigens. II. The glomerular basement membrane, actomyosin, and fibroblast surface antigens in normal, diseased, and transplanted human kidneys. *Am J Pathol* 90:71–88, 1978
27. Simonson MS, Culp LA, Dunn MJ: Rat mesangial cell-matrix interactions in culture. *Exp Cell Res* 184:484–498, 1989
28. Schafer BW, Heizmann CW: The S100 family of EF-hand calcium-binding proteins: functions and pathology. *TIBS* 21:134–140, 1996
29. Zimmer DB, Cornwall EH, Landar A, Song W: The S100 protein family: History, function, and expression. *Brain Res Bull* 37:417–429, 1995
30. Calabretta B, Battini R, Kazmarek L, de Riel JK, Baserga R: Molecular cloning of the cDNA for a growth factor-inducible gene with strong homology to S-100, a calcium-binding protein. *J Biol Chem* 261:12628–12632, 1986
31. Filipek A, Kuznicki J: Calyclin—from basic research to clinical implications. *Acta Biochim Polonica* 40:321–327, 1993
32. Kuznicki J, Kordowska J, Puzianowska M, Wozniewicz BM: Calcyclin as a marker of human epithelial cells and fibroblasts. *Exp Cell Res* 200:425–430, 1992
33. Floege J, Radeke HR, Johnson RJ: Glomerular cells *in vitro* versus the glomerulus *in vivo*. *Kidney Int* 45:360–368, 1994
34. Johnson RJ, Floege J, Yoshimura A, Iida H, Couser WG, Alpers CE: The activated mesangial cell: A glomerular "myofibroblast"? *J Am Soc Nephrol* 2(10 Suppl):S190–S197, 1992